The concentrations and distributions of phytic acid phosphorus and other mineral nutrients in wild-type and *low phytic acid* Js-12-LPA wheat (*Triticum aestivum*) grain parts

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Abstract: Concentrations of P, phytic acid (*myo*-inositol hexa*kis*phosphate, IP_6), and other mineral storage elements were studied in wild-type and *low phytic acid* (*lpa*) genotype Js-12-LPA wheat (*Triticum aestivum* L.) embryos and rest-of-grain fractions. Environmental scanning electron microscopy images revealed a decreased average size and an increased number of aleurone layer globoids in *lpa* grains compared with the wild type. Energy-dispersive X-ray analyses of unfixed aleurone layer and scutellum cell cytoplasm revealed mainly C, O, P, K, and Mg in both grain types. The starchy endosperm contained virtually no P, K, or Mg, demonstrating no shift of mineral nutrients to that compartment. Scanning transmission electron microscopy – energy-dispersive X-ray analyses of scutellum and aleurone layer globoids in both genotypes revealed that P, K, and Mg were the main mineral nutrients in globoids with low amounts of Ca, Fe, and Zn. Traces of Mn were only in scutellum globoids. Total P was similar between genotypes for the rest-of-grain fractions, which are 97% of grain mass. The main inositol phosphate was IP_6, but a small amount of IP_5 was present. Both *lpa* grain fractions exhibited major reductions in IP_6 compared with the wild type and a threefold increase in inorganic P. The concentration of K decreased in both fractions, while Ca increased 25% in the Js-12-LPA rest-of-grain compared with the wild type. The lack of large differences in mineral concentration and distribution between the wild type and Js-12-LPA indicates that there is no direct role of localization of IP_6 synthesis in mineral distribution.

Key words: inositol phosphates, *low phytic acid*, phosphorus, *Triticum aestivum*, wheat, grains.

Résumé : Les auteurs ont étudié les teneurs en phosphore (P), en acide phytique (*myo*-inositol hexa*kis*phosphate, IP_6) et en d'autres éléments minéraux accumulés, chez les embryons et les autres fractions du grain de blé (*Triticum aestivum* L.) de type sauvage (WT), ainsi que chez ceux du phénotype *faible en acide phytique* (*lpa*), Js-12-LPA. Les images en microscopie par balayage électronique environnementale révèlent une diminution de la dimension moyenne et une augmentation du nombre des globoïdes des couches d'aleurone, chez les grains *lpa,* comparativement aux témoins. Les analyses par énergie dispersive des rayons X, de la couche d'aleurone non fixée, et du cytoplasme des cellules du scutellum, montrent surtout des C, O, P, K, et Mg, chez les deux types de grains. L'endosperme riche en amidon ne contient virtuellement pas de P, K, ou Mg, ce qui démontre qu'il n'y pas de déplacement des nutriments minéraux vers ce compartiment. Les analyses en microscopie électronique à transmission par balayage par énergie dispersive des rayons X, du scutellum et des globoïdes de la couche d'aleurone, chez les deux génotypes, révèlent que les P, K et Mg constituent les principaux minéraux des globoïdes avec de petites quantités de Ca, Fe et Zn. On ne retrouve des traces de Mn que dans les globoïdes du scutellum. Le P total est semblable entre les génotypes dans les autres fractions du grain, qui constituent 97 % du grain. Le principal phosphate d'inositol est le IP_6, mais on observe de petites quantités de IP_5. Les deux fractions du grain du *lpa* affichent de fortes réductions du IP_6, comparativement à celles du WT, et une augmentation du triple du P inorganique. La teneur en P diminue dans les deux fractions, alors que le Ca augmente de 25% dans les autres parties du grain du Js-12-LPA, comparativement à celles du WT. L'absence de fortes différences de la teneur et de la distribution des minéraux, entre le Js-12-LPA et le WT, indique qu'il n'y pas de rôle direct de la localisation de la synthèse de IP_6, dans la distribution minérale.

Mots clés : phosphates d'inositol, *faible en acide phytique*, phosphore, *Triticum aestivum*, grains.

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Introduction

Cereal grains comprise nearly 70% of the global crop production of seeds, grains, and fleshy fruits containing seeds (Lott et al. 2000, 2002). Maize, wheat, rice, and barley comprise over 90% of the nearly two billion metric tonnes of cereals harvested globally each year (Lott et al. 2000, 2002). The phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakis-phosphate, or IP_6) contained in the grains of those four cereals har-

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vested each year is estimated to be over 16 million metric tonnes with the P component being 5.6 million metric tonnes (Lott et al. 2000, 2002). Phytic acid is the main P storage compound in most seed and grains and, depending on the species, may form up to 4.7% of seed dry mass. In most seeds and grains, phytic acid forms 50%–80% of the total P content (Cosgrove 1966; IUPAC-IUB 1968; Loewus and Murthy 2000; Lott et al. 2000). The phosphorus component of phytic acid will be termed IP_6-P in this report.

In seeds and grains, the negative charges on phytic acid are usually charge-balanced by cationic elements such as K, Mg, Ca, Fe, Zn, and Mn, which are important plant mineral nutrients (Lott 1984; Wada and Lott 1997). The salt formed by phytic acid and these cations is called phytate and it is located in vacuoles, commonly as dense spherical particles called globoids (Lott 1984). Phytate is thus a vital store of mineral nutrients used for growth and establishment of seedling plants.

Phytic acid is often believed to be an antinutritional substance because the digestive tracts of monogastric animals including humans, swine, and poultry are not able to digest this compound (Thompson 1993). In addition to the P in phytic acid not being bioavailable to many animals, phytic acid is such an effective chelator that elements such as Fe, Zn, and Ca are bound up and excreted with it in faeces. Possible negative effects of phytic acid in seeds and grains used as food by humans and many domestic animals include (*i*) reduced bioavailability of several important mineral nutrients, potentially causing health problems (Zhou et al. 1992; Thompson 1993; Sathe and Reddy 2002), (*ii*) increased pollution of ground and surface waters from P in manure (Raboy et al. 2001), and (*iii*) increased input costs owing to the necessity of adding mineral supplements to animal feeds (McWard 1969).

One approach to counter the negative effects of phytic acid in seeds and grains used for food has been the selection of *low phytic acid* (*lpa*) genotypes that have approximately normal P concentrations but reduced concentrations of phytic acid. Such *lpa* genotypes now exist for the most important legume crop, soybean (Wilcox et al. 2000; Hitz et al. 2002; Oltmans et al. 2005), and for the major cereal crop plants, wheat (Guttieri et al. 2004), rice (Larson et al. 2000), maize (Raboy and Gerbasi 1996; Raboy et al. 2000; Raboy et al. 2001), and barley (Larson et al. 1998; Hatzack et al. 2001; Dorsch et al. 2003).

In this study, P and plant mineral nutrient deposition in wild-type (WT) and *lpa* genotype Js-12-LPA mature wheat grains were compared. This wheat *lpa* genotype has about a 38% reduction in whole-grain phytic acid P (Guttieri et al. 2004). Questions to be answered were (*i*) did the *lpa* genotype bring about any significant changes in the concentrations of elements P, K, Mg, and Ca in the embryo and rest-of-grain fractions, (*ii*) did the *lpa* genotype bring about any tissue-specific changes in element concentrations and localizations in the grains compared with WT, (*iii*) did the *lpa* mutations present in this genotype influence the inositol phosphates in different grain fractions in the same way, and (*iv*) did the *lpa* genotype Js-12-LPA bring about structural or element content changes in the mineral nutrient storage particles called globoids?

Materials and methods

Wheat grains

The wheat (*Triticum aestivum* L. 'Tetonia') grains used in this research were provided by Dr. Ed Souza (Aberdeen Research and Extension Center, University of Idaho, Moscow, Idaho). Both WT and Js-12-LPA grains were harvested at Tetonia, Idaho, in 2001 as described in Guttieri et al. (2004). Grain was produced from a very large number of plants grown under agronomic conditions and practices standard for this region of Idaho. The grains used in this study were from a 2 kg sample of each genotype taken from a much larger pooled sample. Only mature, normal-appearing, undamaged grains were used throughout this research.

Environmental scanning electron microscopy investigation

Environmental scanning electron microscopy (ESEM) imaging was performed to determine the structure of selected cell regions in tissues that received no metal coating or chemical treatment. Five typical grains of both WT and Js-12-LPA were initially cut transversely at midgrain. The grain half not containing the embryo was used to study the aleurone layer, while the embryo-containing grain half was cut longitudinally to expose the midgrain scutellum region of the embryo. The anatomy of a wheat grain is given in Hoseney (1986). Samples mounted on aluminum stubs were viewed in an Electro-Scan model 2020 ESEM operating at 20 kV.

Scanning transmission electron microscopy investigation

Scanning transmission electron microscopy (STEM) was used to determine elemental content and ultrastructure of globoids in selected cell types and to determine the ultrastructure of aleurone globoids. Eight typical grains of each genotype were soaked in 90% ethanol (EtOH) for 1 h followed by removal of the embryo. Samples of scutellum and midgrain aleurone layer cells were prepared with the low-water content procedure of Lott et al. (1984), which permits resin infiltration while retaining phytate. Samples were soaked in 80% EtOH, 100% EtOH, and then propylene oxide for 24 h, respectively. Samples were infiltrated through a propylene oxide-Spurr's resin series for 7 d before embedding and resin polymerization. No electron-dense stains were used, as the globoids were naturally electron dense. To retain elements, sections 1.5 μm thick were cut dry using a glass knife in a Reichert-Jung Ultracut Ultramicrotome, transferred to Formvar-carbon-coated 100-mesh copper grids, flattened with a drop of 100% EtOH, and viewed in a JEOL 1200 EX-II TEMSCAN microscope operating at 80 kV.

Energy-dispersive X-ray analyses

Analyses were performed on unfixed scutellum, starchy endosperm, and midgrain aleurone layer cells using a 1/4 raster at 1000× magnification on an Electroscan 2020 ESEM operated at 20 kV. Globoids from aleurone layer and midgrain scutellum cells were studied using STEM – energy-dispersive X-ray (EDX) analysis. Elements present in selected cell structures were analyzed with PGT IMIX EDX analysis systems (Princeton Gamma Tech, Princeton, New Jersey) attached to a JEOL 1200 EX-II TEMSCAN oper-

ated at 80 kV with a beam current of approximately 54 μA. Owing to differences in detector capabilities, C and O peaks are present in ESEM–EDX analyses but lacking in STEM–EDX analyses. Working conditions were kept constant for all samples. Spectra were saved if the count rate remained above 1000 counts per second. A minimum of 110 spectra were collected for each sample tissue and genotype. Peak-to-background ratios were calculated for P, K, Mg, Ca, Fe, Mn, and Zn as in West and Lott (1993). Corrections for overlapping peaks (such as the overlap of Ca by K) were done as per West and Lott (1993). Determination as to whether or not a globoid contained traces of Ca, Fe, Zn, and Mn was done with the Beecroft and Lott (1996) procedure that takes into account fluctuations around the background subtraction line. The peak-to-background ratios between the same tissue of the two genotypes were tested for significant differences using MINITAB's two-sample *t* test ($P = 0.05$).

Sample preparation for elemental analyses

To improve the precision of embryo removal, WT and Js-12-LPA grains were soaked in 90% EtOH for 2–3 h and then separated into "embryo" and "rest-of-grain" fractions. Less than 1% of mineral elements were lost to the soaking solution. Whole grains and grain fractions were then milled using a stainless steel grinder until all pieces could pass through a 1 mm screen. Embryos were ground only for the total P colorimetric assay; otherwise, they were used whole.

Phytic acid P HPLC assay

Duplicate 15 mg of embryo samples or 250 mg of ground rest-of-grain tissue were placed into small test tubes. One or 2 mL of 0.4 mol HCl/L containing 10% sodium sulphate was added to each embryo or rest-of-grain tube, respectively. The samples were homogenized six times for a total of 3 min and then centrifuged at 16 000*g* in 2.0 mL microcentrifuge tubes for 8 min. The supernatants were removed and stored in fresh microcentrifuge tubes until 200 μL of each sample was assayed by HPLC using the method of Dorsch et al. (2003) with the revision that the metal dye detection colorimetric agent contained 0.015% $FeCl_3$ – 0.15% sulphosalicylic acid.

Inorganic P colorimetric assay

Duplicate 15 mg of embryo samples or 250 mg of ground rest-of-grain tissue were placed into small test tubes and 1 or 2 mL of 12.5% trichloroacetic acid containing 0.2 mol $MgCl_2$/L was added, respectively. The samples were homogenized, centrifuged, and stored frozen as described for phytic acid P (IP_6-P) analyses. Triplicate samples were assayed colorimetrically using the procedure established by Chen et al. (1956). One hundred microlitres of supernatant was brought to a volume of 8.0 mL by adding 3.9 mL of double-distilled water and 4.0 mL of Chen's reagent. The samples were allowed to stand for 2 h at room temperature before reading in a Beckman DU-650 spectrophotometer at 820 nm. Appropriate standards were used for calibration.

Total P colorimetric assay

Duplicate 25 mg of ground embryo tissue samples or 150 mg of ground rest-of-grain tissue were added to 50 mL glass digestion tubes. To each embryo or rest-of-grain tube was added 1.0 or 2.0 mL of concentrated sulphuric acid, respectively. The samples were heated to 120 °C on a hotplate with addition of six or seven drops of 30% H_2O_2 every 30 min until the samples were completely digested. The samples were allowed to cool to room temperature and diluted with double-distilled water to 6.25 mL for the embryo samples and 12.5 mL for the rest-of-grain samples. Triplicate 100 μL samples were assayed according to Chen et al. (1956) as described above.

Elemental concentration analysis

Percent moisture was determined by weighing samples of whole grains, rest-of-grains, and embryos before and after heating in an oven for 2 h at 130 °C (Roberts and Roberts 1972). Quantitative analysis results are presented on a dry mass basis.

Samples for K and Mg analysis were wet-ashed using a Kjeldahl digester. Tubes containing 3 mL of concentrated nitric acid and 0.5 mL of concentrated sulphuric acid were heated until dense white fumes appeared. Once cooled, four or five drops of 30% H_2O_2 were added to each tube to decolour the solution. Tubes were then heated to fuming again to complete the digestion. Calcium analysis samples were dry-ashed to prevent suppression problems resulting from sulphuric acid interference. Samples were charred on a hotplate and then heated in a furnace at 550 °C for 4 h. Ash was treated with dilute acids as described in Gorsuch (1970) and Ockenden and Lott (1986). After centrifugation, all supernatants were diluted to the appropriate volume with 2% HNO_3 containing 1000 μg Cs^+/mL and 2000 μg La^{3+}/mL.

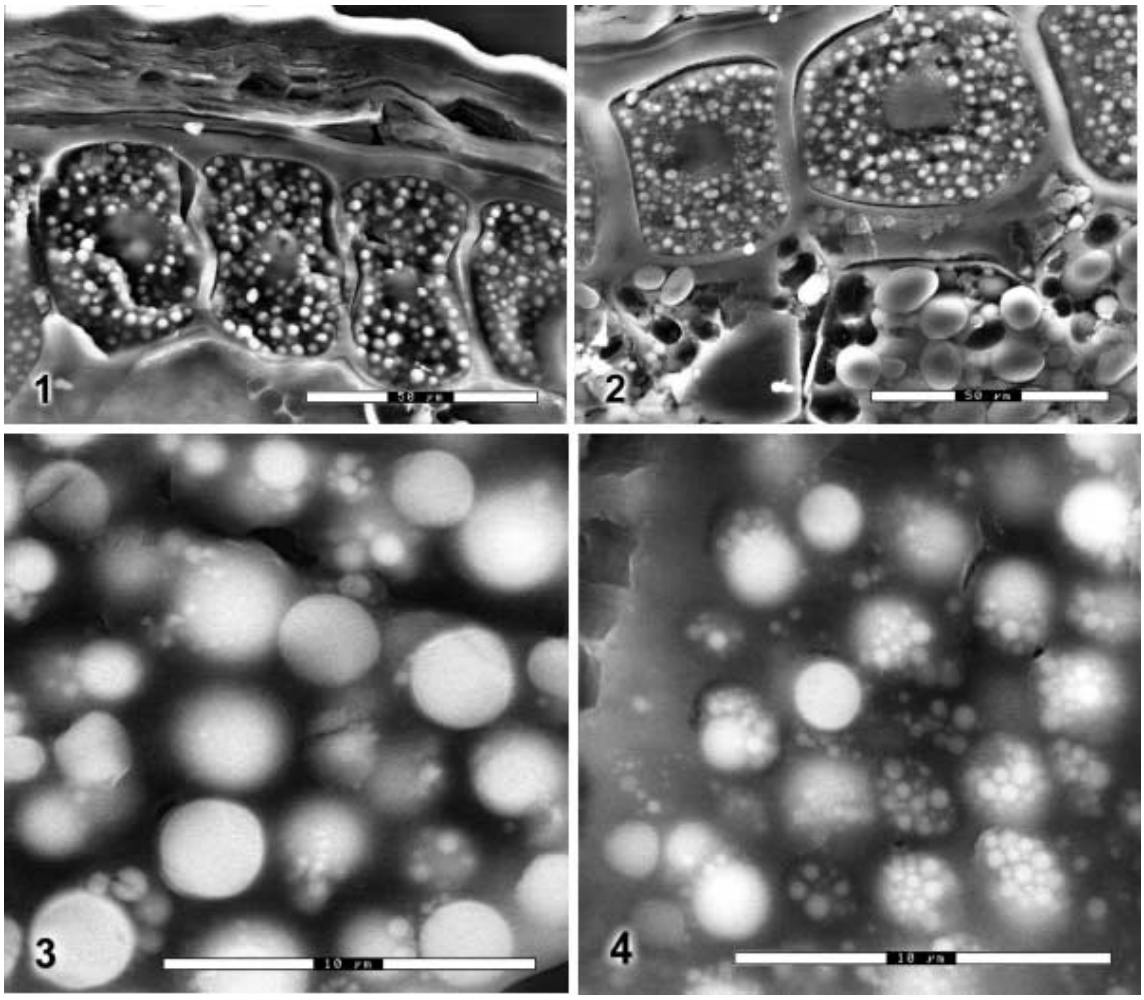
Potassium, Mg, and Ca concentrations were measured via flame atomic absorption spectroscopy (FAAS) with an air–acetylene flame in a Varian Spectr AA 220 (Varian Techtron Pty. Limited, Springvale, Australia) using parameters described in the operators manual. Samples were compared with purchased FAAS standards. Quantitative values were tested for differences using a two-sample *t* test of equal variance ($P = 0.05$).

Results

Electron microscopy investigation

ESEM investigation revealed that in both WT and Js-12-LPA grains, the aleurone layer was generally one cell thick and surrounded by thick cell walls (Figs. 1–4). Globoids appeared as white or pale grey–white spheres in these images. The approximately spherical protein storage vacuoles that contain the globoids could often be seen. At low magnification (Figs. 1 and 2), aleurone cell cytoplasm appeared rather similar in both grain types, but at higher magnification (Figs. 3 and 4), differences in the size and number of globoids were evident. Both large globoids (with diameters of up to 3 μm) and progressively smaller diameter globoids were present in each grain genotype (Figs. 3 and 4). The total number of globoids per Js-12-LPA aleurone layer cell was greater than in the WT cell, but the size of most globoids in Js-12-LPA cells was much smaller than that in WT. In Js-12-LPA aleurone layer cells, globoids frequently appeared in clusters. ESEM investigation of scutellum globoids between WT and Js-12-LPA samples revealed a simi-

Figs. 1–4. ESEM images of dry-cut WT (Figs. 1 and 3) and Js-12-LPA (Figs. 2 and 4) mature wheat grains. All images are of midgrain aleurone layer cells. Scale bars = 50 μm for Figs. 1 and 2; scale bars = 10 μm for Figs. 3 and 4.



lar range of sizes as compared with the aleurone layer cells (images not shown).

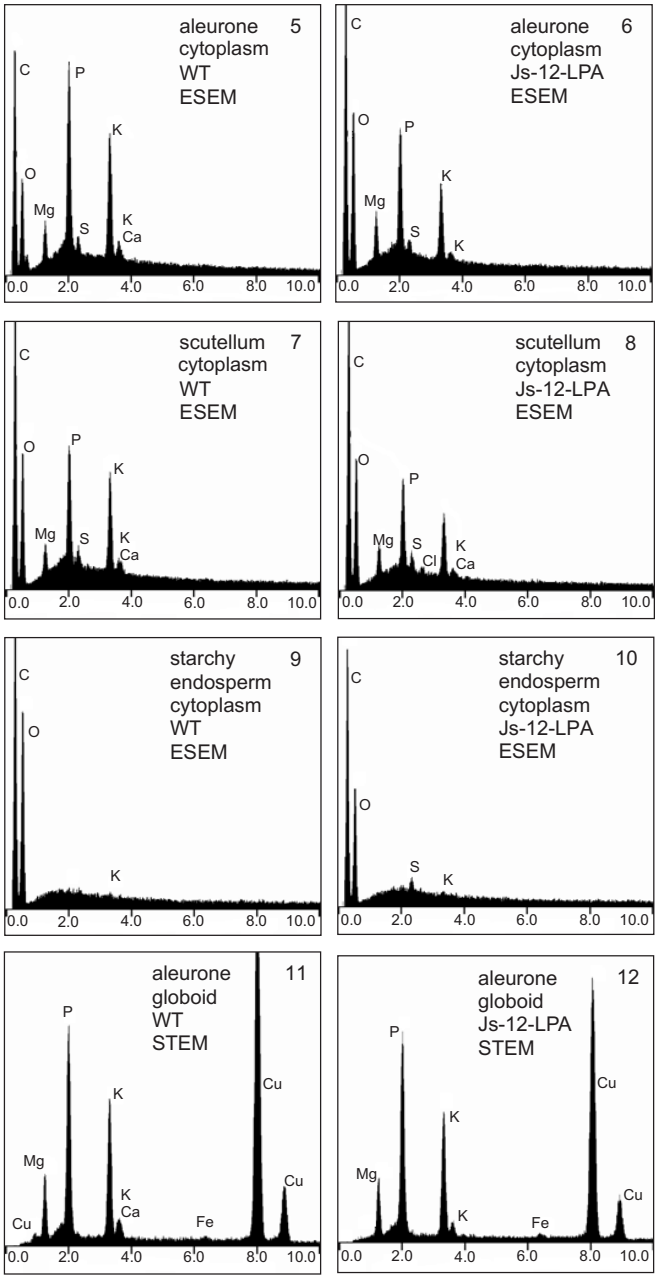
EDX analysis

ESEM–EDX analysis was performed to determine relative element concentrations (Figs. 5–10). Although not strictly quantitative, it is valid to compare peak heights to determine the relative proportions of elements between samples, as instrument operating conditions were kept standard and vertical axes were standardized between grain genotypes. As expected for biological tissue, there were major peaks for C and O. Of the other elements detected, P was present in the highest amount in both aleurone and scutellum tissues for both WT and Js-12-LPA grains followed by K, Mg, and S (Figs. 5–8). Traces of Cl were found in Js-12-LPA scutellum tissue (Fig. 8), and some Ca was often found. In both aleurone and scutellum cell cytoplasm, P and K concentrations often were lower in the Js-12-LPA grains compared with WT (compare respective peak heights of Figs. 5 and 7 with those of Figs. 6 and 8), while Mg and S peak heights were similar to those in WT. In addition to C and O peaks, the starchy endosperm had very small peaks for K in both grain genotypes and a small S peak in the Js-12-LPA tissue (Figs. 9 and 10).

STEM–EDX analysis of aleurone globoids in both grain types revealed that P, K, and Mg were present in large amounts, with P being the greatest followed by K and then Mg (Figs. 11 and 12). Small amounts of Fe and Ca were detected in many aleurone globoids. Relative proportions of P, K, Mg, and Fe were similar between WT and Js-12-LPA aleurone globoids.

Results for WT and Js-12-LPA aleurone and scutellum layer globoids, presented as peak-to-background ratios (Table 1), provide a useful method of comparing different samples. Of the main elements, P, K, and Mg, the largest difference between WT and Js-12-LPA samples was that the P content of the Js-12-LPA scutellum globoids was about 20% less than that of the WT. The K content of the Js-12-LPA scutellum globoids was lower than that of the WT, but the levels were similar in the aleurone globoids of the two grain types. The concentration of Mg increased in Js-12-LPA aleurone globoids but was similar in scutellum globoids relative to the WT. There were increases in Ca content in both aleurone and scutellum globoids in the Js-

Figs. 5–12. EDX analysis spectra of rastered cytoplasm areas of dry-cut WT and Js-12-LPA wheat grains using ESEM (Figs. 5–10) or electron-dense globoids in sections viewed in STEM mode (Figs. 11 and 12). Horizontal axes show X-ray energy levels (keV) from 0.0 to 10.0. Vertical full scales are 2500 (Figs. 5–8) and 2000 (Figs. 9–12). Each spectrum presented is typical of the given grain type and tissue. The X-ray detector used for ESEM analysis allows measurement of C and O (peaks seen on the left side of the spectra). In STEM mode, X-rays from C and O are eliminated by the detector's beryllium window. Copper grids used for STEM sections gave artifact peaks for Cu at 8.04 keV, K_α, and 8.91 keV, K_β. Principal X-ray lines of elements and energies (keV) are as follows: C, K_α 0.28; O, K_α, 0.52; Mg, K_α, 1.25; P, K_α, 2.02; S, K_α, 2.31; Cl, K_α, 2.62; K, K_α, 3.31; K, K_β, 3.59; Ca, K_α, 3.69; Ca, K_β, 4.01; Fe, K_α, 6.40; Zn, K_α, 8.63. The overlap of the main K_α peak for Ca by the secondary K_β peak of K makes determination of traces of Ca difficult.



12-LPA samples compared with WT. Manganese was virtually absent from aleurone globoids, while Fe and Zn traces were present in both tissues in both grain types. The Zn content was found to be lower in the Js-12-LPA grains in both aleurone and scutellum globoids compared with WT.

In both grain types, Ca and Mn peak-to-background ratios were higher in scutellum globoids than in aleurone globoids (Table 1). Peak-to-background ratios for the other five elements measured by EDX analysis revealed that in 8 of 10 cases, scutellum globoids had lower peak-to-background ratios than did aleurone globoids (Table 1), and in two cases, there was no significant difference.

To further compare any differences between grain genotypes for the elements present in globoids in trace quantities, the frequency of occurrence of Ca, Mn, Fe, and Zn in any given EDX spectrum was calculated. There was an increase in the occurrence of Ca in WT and Js-12-LPA scutellum globoids (94% and 100%, respectively) compared with aleurone globoids (72% and 67%). In both genotypes, the frequency of Mn in the aleurone globoids was found to be near zero, yet Mn was present in nearly all scutellum globoids. Fe was present in 98%–100% of all globoids in both grain genotypes and both tissues. Zinc was present in the Js-12-LPA aleurone globoids less frequently (10%) than in the WT globoids (32%).

Grain masses

Masses were not significantly different between WT and Js-12-LPA genotypes in whole grains (35.58 versus 35.50 mg, respectively), embryos (1.17 versus 1.08 mg), and rest-of-grain (35.72 versus 35.54 mg). The embryo was found to be 3% of grain total mass in both types. Whole-grain mass values fall within published ranges for wheat grains (Hoseney 1986). The sum of the masses of the embryo and rest-of-grain fractions compared with the original whole-grain mass demonstrated that there was little change in mass resulting from the separation process.

Element concentrations and inositol phosphate analyses

The total P concentration in rest-of-grain portions was not significantly different between WT and Js-12-LPA grains but was lower in the Js-12-LPA embryos (Table 2). The concentrations of IP_6-P, which is the P component of IP_6, were 56% and 37% lower, respectively, in the embryo and the rest-of-grain portions of the Js-12-LPA mutant than in the WT. Inorganic P (P_i-P) concentrations in Js-12-LPA embryos and the rest-of-grain portions were three times the P_i-P found in the WT corresponding grain parts. The total concentrations of IP_6-P in the wheat grain were 25–60 times higher than the amount of IP_5-P.

Of the three major cationic elements analyzed, namely K, Mg, and Ca, the concentration of K was the highest in both grain fractions in both grain types, with Mg the next highest and Ca the lowest (Table 3). The embryo had a markedly higher concentration of each element measured by FAAS than the corresponding rest-of-grain fraction. Compared with WT, the Js-12-LPA grains had lower K and Ca concentrations in the embryo and lower K but higher Ca in the rest-of-grain fractions (Table 3). Magnesium concentrations were not significantly different in both grain fractions between the two genotypes. The Js-12-LPA grains had decreased Ca con-

Table 1. Mean peak-to-background ratios for P, K, Mg, Ca, Mn, Fe, and Zn from EDX analysis of globoids in aleurone cells and scutellum of WT and Js-12-LPA genotype wheat grains comparing the same tissues between different wheat types.

Seed tissue and type	P	K	Mg	Ca	Mn	Fe	Zn
Aleurone							
WT	8.21±1.20a	9.61±2.00a	4.92±1.71a	0.33±0.25a	0.01±0.09a	0.40±0.18a	0.28±0.33a
Js-12-LPA	8.69±1.37b	10.13±1.89a	5.45±1.80b	0.52±0.49b	0.02±0.10a	0.41±0.24a	0.06±0.12b
Scutellum							
WT	8.10±1.37a	7.75±1.67a	4.32±1.14a	0.73±0.48a	0.38±0.31a	0.27±0.14a	0.13±0.16a
	NSD	L	L	H	H	L	L
		19.5%	12.3%	223.6%	2951.2%	33.7%	52.4%
Js-12-LPA	6.46±1.85b	6.97±1.65b	4.12±1.81a	1.11±0.41b	0.45±0.25a	0.33±0.18b	0.08±0.12b
	L	L	L	H	H	L	NSD
	25.6%	31.2%	24.4%	212.1%	3037.4%	20.1%	

Note: Values are means ± SD. For all seed tissues and types, $N = 110$ for each value given. For a given element and tissue, values followed by different letters are significantly different ($P = 0.05$). L and H indicate that peak-to-background ratios in the scutellum globoids were lower or higher, respectively, than those in the aleurone globoids from a given genotype, where significant differences were found ($P = 0.05$). NSD, no significant difference. Percentages are of an increase or reduction in the mean element peak-to-background ratio of scutellum globoids compared with aleurone globoids.

Table 2. Total P, IP_6-P, P_i-P, and IP_5-P levels in wheat grain fractions.

Genotype and grain fraction	Total P (mg/g)	IP_6-P (mg/g)	P_i-P (mg/g)	IP_5-P (mg/g)
WT embryo	11.283±0.458a	9.112±0.712a	0.660±0.032a	0.150±0.211
Js-12-LPA embryo	9.708±0.004b	4.031±0.126b	1.836±0.059b	ND
WT rest-of-grain	2.847±0.029a	2.444±0.067a	0.224±0.013a	0.083±0.016a
Js-12-LPA rest-of-grain	2.860±0.057a	1.550±0.207b	0.774±0.080b	0.061±0.005a

Note: Duplicate samples were analyzed, and all values are means ± SD, with units of mg P/g dry mass. IP_6-P, Pi-P, and IP_5-P are the P (atomic mass 31) components of phytic acid, inorganic P, and inositol pentaphosphate, respectively. For a given grain fraction, pairs of values within a column with the same letter are not significantly different based on a two-sample *t* test of equal variance ($P = 0.05$). ND, a result below the detection limit. The lower detection limits for IP_5-P or IP_6-P with the methods used here were 0.100 and 0.050 mg/g for embryo and rest-of-grain samples, respectively.

centration in the embryo but had higher concentration in the rest-of-grain fraction than the WT grains (Table 3). The embryo had significantly higher concentrations of K, Mg, and Ca than the corresponding rest-of-grain by a factor of about 3, 2, and 1, respectively. However, on a per part basis (Table 3), the amount of K, Mg, and Ca in the rest-of-grain was much higher than in the embryo. There were no per part differences in Mg content in either fraction but there were per part differences in K and Ca content (Table 3). WT embryos contained more K and Ca than Js-12-LPA embryos.

Discussion

Electron microscopy investigation

The Js-12-LPA genotype did not alter the thickness or the number of cells in the aleurone layer nor did it result in a major shift of elements to the starchy endosperm. In WT grains, the storage of P, K, and Mg is in the embryo and aleurone layer and that same pattern occurs in *lpa* grains. The Js-12-LPA genotype did result in an overall reduction in globoid size. The reduction in aleurone layer globoid size is in agreement with the trend of *lpa* mutations producing greater numbers of smaller globoids in other grains, such as rice (Liu et al. 2004) and barley (Ockenden et al. 2004). Lott et al. (1994) hypothesized that the globoids form through the cross-linking of phytic acid molecules through divalent and (or) trivalent cations such as Mg^{2+} and Fe^{3+}. According to this hypothesis, then, the reduction in globoid size is to be expected owing to the level of phytic acid within the grains being significantly reduced, decreasing the number of phytic acid molecules available for cross-linking. It is likely that the decrease in IP_6-P is balanced by an increase in P_i in the same subcellular compartment. Since each IP_6 molecule has 12 negative charges, the chances for molecule to molecule cross-linking by cations are higher than it would be for P_i alone.

Although the STEM–EDX analysis of aleurone layer globoids revealed some differences, very large scale differences were not found in the elemental composition of the globoids, suggesting that only their size and number were affected by the Js-12-LPA genotype. Size is more important than globoid number, however, as the volume increases exponentially with increasing diameter. One large globoid with a diameter of 2.75 μm, as occurs in WT aleurone cells, has the same volume as 55 globoids with a diameter of 0.75 μm (Ockenden et al. 2001). The number of globoids per protein-storing vacuole was greater in Js-12-LPA than in WT but not by the number of globoids that the calculation above re-

Table 3. Element concentrations and element amounts per part in wheat WT and Js-12-LPA grain fractions.

Element	Grain type	Embryo (μg/g)	Rest-of-grain (μg/g)	Embryo (μg)	Rest-of-grain (μg)
K	WT	11962.9±795a	3025.1±24.4a	12.43±0.82a	96.47±0.77a
	Js-12-LPA	9966.0±883b	2660.2±90.8b	9.93±0.89b	84.52±2.89b
Mg	WT	4821.4±539a	1711.7±56.3a	5.01±0.56a	54.58±1.80a
	Js-12-LPA	4195.1±289a	1692.3±16.7a	4.18±0.29a	53.77±0.54a
Ca	Wt	710.6±17.1a	306.1±5.29a	0.740±0.017a	9.760±0.169a
	Js-12-LPA	660.8±14.1b	382.7±6.52b	0.656±0.014b	12.162±0.208b

Note: For a given element and a given fraction, pairs of mean values with the same letter are not significantly different based on a two-sample *t* test of equal variance ($P = 0.05$). Triplicate samples were analyzed, and all values are given in concentrations of micrograms per gram dry mass ± SD or micrograms of element/grain fraction ± SD.

quires. Thus, while globoid number was increased, the total volume was lower. The factors influencing the formation of globoids are poorly understood.

The presence and levels of P, K, and Mg in the cytoplasmic EDX spectra of the WT grains are consistent with those found previously using other methods (Heard et al. 2002). Calcium, Fe, and Zn are present, in most cases, in globoids in both the aleurone layer and the scutellum cells in both genotypes, although in very small amounts. The peak-to-background results revealed that Ca is present more frequently and in greater quantity in the embryo (scutellum) than in the aleurone layer, with a larger increase in the Js-12-LPA embryo versus the aleurone layer than in WT. Comparison of ESEM–EDX analysis of unfixed areas of cell cytoplasm with STEM–EDX analysis of individual globoids in sections of embedded tissue demonstrated two points: (*i*) that the low water content specimen preparation procedure retained elements of interest in both grain types and (*ii*) that almost all X-ray signals came from globoids.

Elemental concentration analyses

The 38% reduction in IP_6-P concentration between the whole-grain WT and Js-12-LPA grains reported for *lpa* wheat (Guttieri et al. 2004) is mirrored in the decrease in IP_6-P that we found for the embryo and rest-of-grain fractions. There was a major difference in the decrease in IP_6-P between the embryo and rest-of-grain portions (56% and 37%, respectively), demonstrating a tissue-specific influence of the Js-12-LPA genotype. IP_5-P was present in both tissue fractions of WT and in the Js-12-LPA genotype rest-of-grain fraction but in very low concentrations relative to IP_6-P. P_i-P increases over WT were 64% for the embryo and 71.6% for the rest-of-grain fraction. Concentrations of P_i-P in the whole grain should be very close to that in the rest-of-grain fraction, since the tiny embryo (3% of dry mass) would not influence whole-grain concentrations to any appreciable amount.

As there was no net change in P concentrations between the two seed genotypes for the rest-of-grain fraction and a slight decrease in total P in the embryos, the decrease in IP_6-P had to be matched in total amounts by an increase in other forms of P including P_i-P and IP_5-P. Although the P_i-P and the IP_5-P increased, the increase was not equal to the IP_6-P decrease, and so a larger proportion of P in the Js-12-LPA grains as compared with the WT may well be in other P compounds. An increase in other forms of P in *lpa* seeds comparable with WT has been reported by Oltmans et al. (2005) for soybean. In various soybean populations, the other P (total P minus the sum of phytate P and P_i) forms 24%–26% and 36%–39%, respectively, for WT and *lpa* seeds (Oltmans et al. 2005). The other P is the sum of non-inorganic P compounds such as nucleic acids, proteins, lipids, and others (Oltmans et al. 2005). If *lpa* genotypes in general result in a significant increase in other forms of P in seeds and grains, further research is needed to determine the compounds that sequester that extra P.

If the amount of K, Mg, or Ca per average embryo is added to the amount of that element per rest-of-grain fraction, a calculated whole-grain estimate for each of these elements can be obtained. Such whole-grain element concentration estimates obtained from the results presented here are comparable with those measured by O'Dell et al. (1972). The concentration of Mg in a WT embryo was similar to that of a Js-12-LPA embryo and the same pattern applied to Mg concentrations in rest-of-grain fractions. The Js-12-LPA and WT estimated whole-grain element concentrations were similar, showing that the decrease in phytic acid did not affect the total concentrations of these three elements. The estimated whole-grain concentrations of K, Mg, and Ca calculated from our results are similar to those reported by Guttieri et al. (2004), who studied whole grains of WT and Js-12-LPA wheat. Guttieri et al. (2004) studied various milled fractions of WT and Js-12-LPA wheat grains but did not use the dissection procedure used in this study to obtain pure embryos. On a per part basis, little variation in Mg was found between the two genotypes for both the embryo and the rest-of-grain fraction, suggesting that no significant redistribution of Mg occurred. On a per part basis, the K contents of both embryo and rest-of-grain fractions were lower in Js-12-LPA than in WT. Calcium content of embryos was lower in Js-12-LPA than in WT but higher in the rest-of-grain fraction. EDX analysis of Js-12-LPA starchy endosperm (Fig. 10) revealed that no major redistribution of P, K, Mg, or Ca occurred into this tissue as a result of the *lpa* genotype.

In conclusion, we investigated possible differences in the ultrastructure, inositol phosphates, and plant mineral nutrients between WT and Js-12-LPA wheat grains and grain fractions. No major changes in grain structure were observed, but mineral nutrient storage globoids were often smaller in Js-12-LPA and larger in WT. No major shifts of elements into the starchy endosperm were observed in the *lpa* genotype, even though the reduced IP_6-P was often balanced by increased P_i. Some tissue-specific effects were ob-

served such as a larger decrease in IP_6-P in the embryo in relation to the rest-of-grain portions. On a per part basis, results were very consistent in that the rest-of-grain fraction, which includes the aleurone layer and forms 97% of the grain mass, had most of the total P as well as formed 80%–87% of the per part amounts of three elements acting as major counterions (K, Mg, and Ca). These results indicate that the localization of IP_6 synthesis in the germ and aleurone plays a minor role in mineral localization in these tissues, even though IP_6 serves as the site of binding of various cationic elements.

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